Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 2011, 23(11) 1858–1864
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Archaeal community structure along a gradient of petroleum contamination in saline-alkali soil

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Received 19 January 2011; revised 16 March 2011; accepted 22 April 2011

Abstract

The response of archaeal communities to petroleum contamination in saline-alkali soil was characterized by analyses of three soil samples with different total petroleum hydrocarbon concentrations. Through the construction and screening of 16S rRNA gene clone libraries based on DNA extracts from these soils, nine distinct phylogenetic groups were identified. Statistical analyses showed that the distribution of archaeal community structures differ significantly along the gradient of petroleum contamination in these three saline-alkali soils. Five phylogenetic groups were dominant in the control soil, two of which were also abundant in the lightly contaminated soil. Four phylogenetic groups were dominant in heavily contaminated soil, one of which was also abundant in the lightly contaminated soil. The halophilic genus of *Haloferax* and the haloalkaliphilic genus of *Natronomonas* were more abundant in heavily contaminated soil. These results suggested that the genera of *Haloferax* and *Natronomonas* may have a role in the natural attenuation of petroleum-contaminated saline-alkali soil.

Key words: archaeal community; petroleum hydrocarbon; saline-alkali soil; 16S rRNA gene clone library

DOI: 10.1016/S1001-0742(10)60640-7

Citation: Wang X X, Han Z, Bai Z H, Tang J C, Ma A Z, He J Z et al., 2011. Archaeal community structure along a gradient of petroleum contamination in saline-alkali soil. Journal of Environmental Sciences, 23(11): 1858–1864

Introduction

In China, oilfields such as Daqing Oilfield, Shengli Oilfield and Dagang Oilfield are located at saline-alkali regions that had been contaminated with petroleum due to spills that occurred during transport and storage of petroleum products (Nie et al., 2009; Tang et al., 2010). The areal extent of contamination is about 10,000 ha (Li et al., 2006). Remediation technologies for petroleum-contaminated soils include biostimulation, bioaugmentation and phytoremediation (Al-Mailem et al., 2010a; Mishra et al., 2001). However, these technologies are difficult to implement and are economically infeasible to remediate such gigantic area of petroleum-contaminated saline-alkali soil. Thus, natural attenuation through microbial degradation is an alternative remediation technology for mitigating petroleum contamination in saline-alkali soils. The role of bacterial and fungal communities in hydrocarbon biodegradation has been reported in literature (Hadibarata and Tachibana, 2010; Haritash and Kaushik, 2009; Wang et al., 2010), but there is a paucity of information on the role of archaeal communities.

Hydrocarbon-degrading Archaea have been isolated from extreme aquatic environments such as coastal waters (Al-Mailem et al., 2010b), saline waters (Tapilatu et al., 2010) and salt marshes (Bertrand et al., 1990). Several Archaea were also reported to be involved in the biodegradation of petroleum hydrocarbons in extreme marine environments (Stetter et al., 1993). Although there is an increasing interest in the function of Archaea in hydrocarbon biodegradation, knowledge about archaeal community structure in petroleum-contaminated soils, particularly in saline-alkali soils, is still limited.

This study compares the archaeal communities presented in three saline-alkali soils with different levels of total petroleum hydrocarbon (TPH) contamination by 16S rRNA gene clone library analysis. The aim of this study was to assess the response of archaeal communities to petroleum contamination in saline-alkali soil, which will allow a better understanding about archaeal diversity in the natural attenuation of petroleum-contaminated saline-alkali soil.

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1 Materials and methods

1.1 Soil sampling and total petroleum hydrocarbon determination

Soil samples were collected from a saline-alkali region at the Dagang Oilfield (38°42′10″N, 117°29′26″E) in China in May 2010. The extent of petroleum contamination was about 3 m^2, and residual petroleum was observed at the soil surface. The soil has been contaminated for 2 years and the petroleum hydrocarbons have therefore undergone weathering. Three soil samples were collected at different distances from the center of the petroleum residue. The distance between two sample locations was less than 1 m to minimize the difference of environmental factors. Soil H was located closest to the center of the remarked petroleum-contamination; Soil L was located slightly farther away from the center; and Soil CK was located farthest from the center without visible petroleum at the soil surface. At each soil sampling location, two soil cores (0–20 cm depth) were collected and combined for further analysis. Soil samples were stored in airtight polyethylene bags and were kept at 4°C until analysis.

Analysis of TPH was performed consistent with the method described by Mishra et al. (2001). TPH in 10 g soil was extracted consecutively with hexane, methylene chloride, and chloroform (20 mL each). All three extracts were pooled and dried at room temperature by allowing the solvents to evaporate under a gentle nitrogen stream in a fume hood. The residual TPH was measured gravimetrically. The TPH was further fractionated into alkane, aromatic, polar N-, S-, O-containing compounds (NSO), and asphaltene fractions on a silica gel column. The TPH was dissolved in *n*-pentane and separated into soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on a silica gel column and eluted with different solvents. The alkane fraction was eluted with 100 mL of hexane, and then the aromatic fraction was eluted with 100 mL of benzene. Finally, the NSO fraction was eluted with 100 mL of methanol and 100 mL of chloroform (Walker et al., 1975).

Soil salinity was determined by evaporating the extract at 180°C at a soil: water ratio of 1:5 (*m*/*V*) (Rhoades, 1996). Soil pH was measured with a pH meter (Delta320, Mettler-Toledo Inc., China) after soil was extracted in 0.01 mol/L $CaCl_2$ at a soil:extractant ratio of 1:5 (*m*/*V*) (Wong et al., 2008).

1.2 DNA extraction and polymerase chain reaction (PCR) amplification

DNA was extracted from the soil samples using the method described by Zhou et al. (1996). The crude DNA extract was electrophoretically separated in an agarose gel (0.8% low-melting agarose, *W*/*V*) and high-molecular weight DNA bands were excised, melted, and purified by using an Omega DNA Gel Recovery Kit (Omega Bio-Tek, Inc., USA) according to manufacturer's instructions. The purified environmental DNA was amplified with an archaeal primer 21F (5′-TTC CGG TTG ATC CTG CCG GA-3′) and 958R (5′-YCC GGC GTT GAM TCC AAT T-3′), as previously described by Delong (1992). Polymerase chain reaction was performed in a total volume of 50 μL, containing 1 μL of each primer, 4 μL of 2.5 mmol/L dNTP mixture, 0.5 μL of 5 U/μL *Taq* DNA polymerase, 5 μL of 10× PCR buffer, 1 μL of template DNA and 37.5 μL ddH_2O. Thermo cycling was performed under the following conditions: initial denaturation at 94°C for 5 min, followed by 25 cycles consisting of 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min, and a final elongation step at 72°C for 10 min. Three independent PCR products, confirmed by agarose gel electrophoresis followed by staining with ethidium bromide, were pooled to minimize the potential effect of PCR biases in single reactions (Bano et al., 2004). The 920 bp fragment was excised from the agarose gel and purified by the same Omega DNA Gel Recovery Kit used in the purification of environmental DNA.

1.3 16S rRNA gene clone library construction and screening

Purified PCR products were ligated into the pGEM-T easy vector (Promega, Germany) following the manufacturer's instructions. *E. coli* DH5α-competent cells were transformed with the ligation products and spread onto Luria-Bertani agar plates with 100 μg/mL ampicillin and X-gal/IPTG on the surface to allow for standard blue and white screening. The sizes of the inserts were verified by amplifying randomly picked clones with vector primers T7 and Sp6.

Amplified ribosomal DNA restriction analysis (ARDRA) was used to distinguish and classify cloned 16S rRNA gene sequences. The PCR products were digested simultaneously with two restriction enzymes, *Msp*I and *Rsa*I, for 3 hr at 37°C in 20 μL reaction volumes containing 10 μL PCR products, 2 μL 10× buffers, 7.5 μL ddH_2O, and 0.5 μL (10 U/μL each) of the restriction enzymes. The restriction fragments were separated electrophoretically in 2.5% (*W*/*V*) agarose gel using 0.5× TBE buffer at 100 V for approximately 1 hr. Clones were grouped according to unique restriction patterns, and representatives of each restriction pattern were sequenced with an ABI 3730 XL DNA analyzer (Applied Biosystems Inc., USA).

1.4 16S rRNA gene clone library analysis

The Bellerophon (Huber et al., 2004) and Mallard (Ashelford et al., 2006) programs were both used to detect PCR-generated chimerical sequences, and suspected chimeras were not included in further analysis. To check the accuracy of ARDRA patterns, sequences were aligned with ClustalW (EMBL, UK) and grouped together based on sequence similarity. Sequences showing 97% sequence similarity were considered to belong to the same operational taxonomic unit (OTU) (Stackebrandt and Goebel, 1994). The remaining sequences were submitted to GenBank (accession numbers: HQ400408 to HQ400568) and were screened against those in this database by using BLASTN (NCBI, USA) to obtain a preliminary phylogenetic affiliation of the clones. The representative sequences

of each OTU were aligned and analyzed using ClustalW. Phylogenetic analysis were performed with MEGA 4.0 (Center for Evolutionary Medicine and Informatics, USA) using neighbor-joining method based on Jukes-Cantor corrected distances with bootstrap value of 1000.

1.5 Statistical analysis

Differences in TPH, soil salinity, and pH values among the samples were tested with one-way analysis of variance (ANOVA) using SPSS software version 13.0 (SPSS Inc., Chicago, USA). The coverage of the clone libraries was estimated according to the method by Good (1953), and the procedure developed by Kemp and Aller (2004) was used to confirm that an asymptotic accumulation curve in each library had been reached. Genotype richness of the clone libraries was estimated using Chao1 estimator and abundance-based coverage estimator (ACE) (Chao, 1987). The abundance-based Jacard and Sørensen similarity indices were used to assess community overlap, which is the probability that two individuals from two communities are members of species shared by both communities (Chao et al., 2005). A statistical comparison of libraries was performed using the Libshuff software program (Schloss et al., 2004).

2 Results and discussion

2.1 Total petroleum hydrocarbon concentrations in soil samples

Soil samples collected at different distances from a petroleum spill showed significantly different ($p < 0.05$) TPH concentrations (Fig. 1). Soil H, located closest to the center of petroleum spill site, had the highest TPH concentration. Whereas soil L, located slightly farther away from the center of the spill, had a lower TPH concentration. The lowest TPH concentration was detected in soil control (CK), the farthest from the center of the spill and considered the most pristine sample. It is believed that hydrocarbon composition has a clear influence on microbial community (Yakimov et al., 2005). Thus the alkane, aromatic, NSO, and asphaltene fractions was further fractionated from TPH, which followed a pattern similar to TPH. Soil H had the highest concentration of these four fractions. Soil L had a lower concentration and soil CK had the lowest concentration. In addition, all soil samples showed a similar pattern of the percentage of these fractions. The alkane fraction was the largest constituent of the TPH over 2 years after the petroleum contamination occurred, followed by the aromatic fraction, the NSO fraction and asphaltene fraction. Predominance of alkane and aromatic fraction suggests that they may have a great influence on soil microbial community.

Soil salinity and pH values, however, were not significantly different among the three samples ($p > 0.05$). Soil salinity was about 1.7% and pH value was about 8.9, which could be classified as highly saline-alkali.

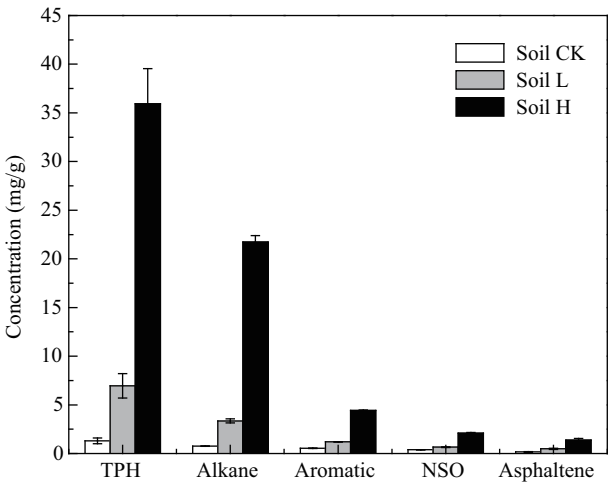

Fig. 1 Composition of petroleum hydrocarbon in saline-alkali soils. CK stands for control soil, L for lightly contaminated soil, and H for heavily contaminated soil. The values shown are means ±SD.

2.2 Estimation of archaeal richness

The soil has been contaminated by petroleum for 2 years, suggesting soil microbial communities have undergone natural selection and acclimation after long-term contamination. Total genomic DNA was extracted from three soil samples, and a 920 bp fragment was obtained by amplification with Archaea-specific primers (Fig. 2). Based on ARDRA and sequence analysis, 68, 51 and 42 OTUs were recovered from soils CK, L, and H respectively. The coverage of these libraries was 83.6%, 86.8%, and 90.5% respectively (Table 1). Two nonparametric richness estimators (Chao1 and ACE) of subsamples from each library reached an asymptotic maximum, thus, proving that these libraries are large enough to yield unbiased estimates (Kemp and Aller, 2004).

The nonparametric estimators, Chao1 and ACE, showed the three soil samples have different archaeal richness (Table 1). Jaccard and Sørensen similarity index among the libraries were low, which indicated there are clear differences among three archaeal communities (Table 2). Significant differences ($p < 0.001$) in the libraries were also confirmed by results of the statistical comparison analysis using the Libshuff program. Correlation analysis showed the TPH concentrations had a significantly negative correlation with Chao1 ($r = –0.877$, $p < 0.05$) and ACE ($r = –0.963$, $p < 0.05$). These results are consistent with previous observations that the extent of petroleum

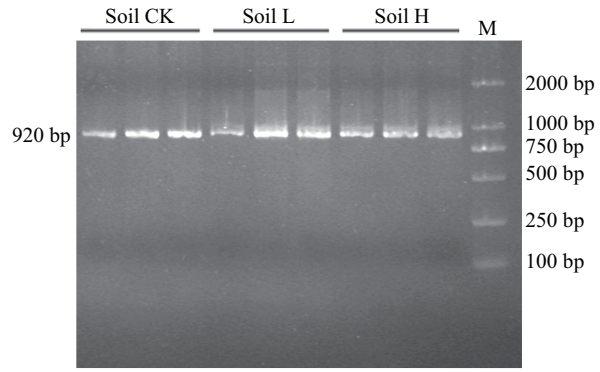

Fig. 2 PCR amplification of archaeal 16S rRNA gene from petroleum-contaminated saline-alkali soils. M: DL2000 DNA marker.

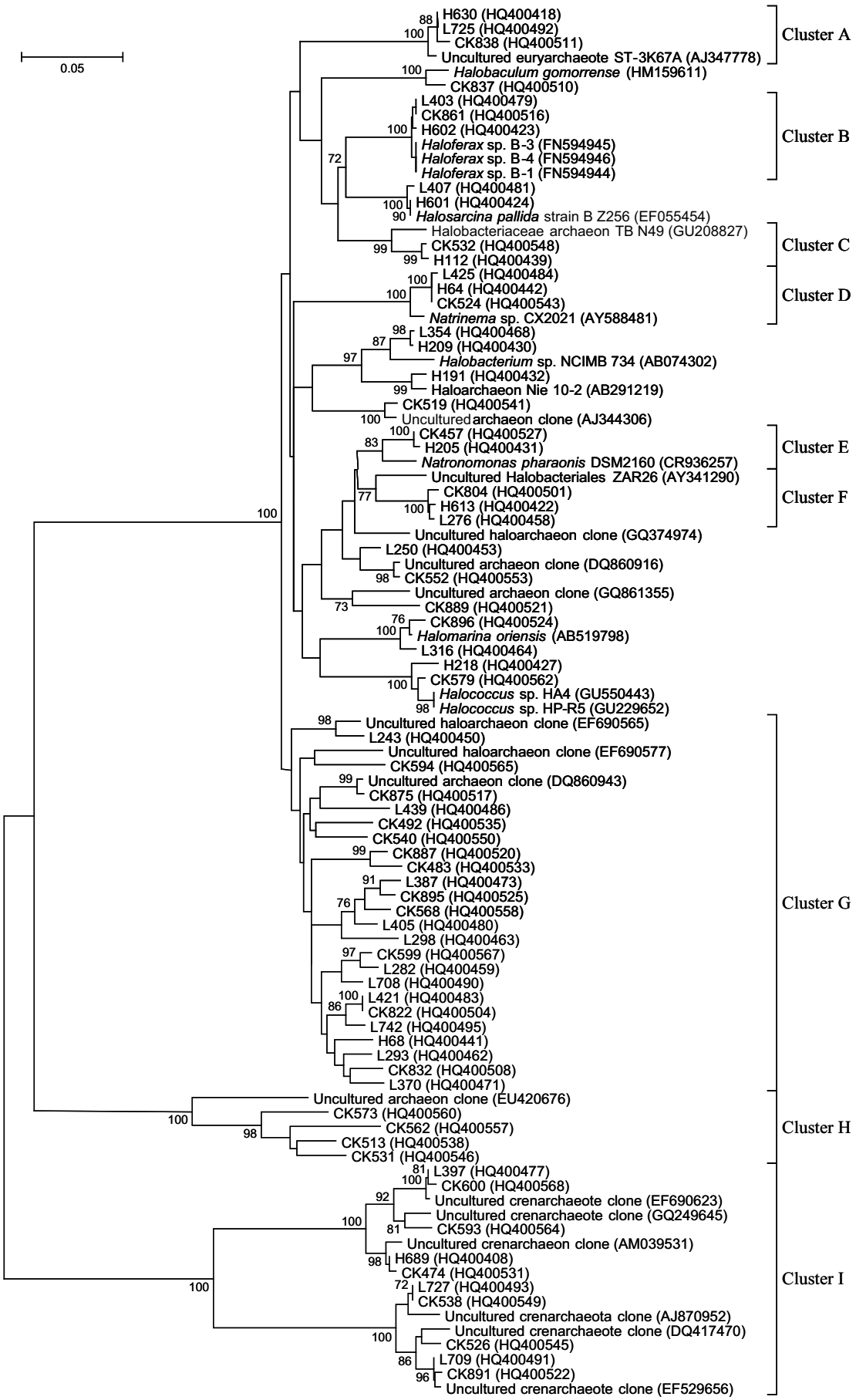

Fig. 3 Phylogenetic tree of archaeal 16S rRNA gene sequences obtained from petroleum-contaminated saline-alkali soils. The tree was constructed with MEGA software 4.0 using neighbor-joining method based on Jukes-Cantor corrected distances with bootstrap value of 1000. Bootstrap values below 70% were not presented. The scale bar represents 5% nucleotide sequence difference.

Table 1 Estimation of archaeal richness in petroleum-contaminated saline-alkali soils

	Soil CK	Soil L	Soil H
Number of clones	207	212	232
Number of OTUs	68	51	42
Coverage	83.6%	86.8%	90.5%
Chaol	105.4	85.4	70.9
ACE	118.5	104.4	79.9

CK stands for control soil, L for lightly contaminated soil, and H for heavily contaminated soil; OTUs: operational taxonomic units; ACE: abundance-based coverage estimator.

Table 2 Comparison of archaeal 16S rRNA gene clone libraries

	Communities compared		
	Soil CK to Soil L	Soil CK to Soil H	Soil L to Soil H
Jaccard similarity index	0.424	0.400	0.598
Sørensen similarity index	0.595	0.571	0.749
p	< 0.001	< 0.001	< 0.001

contamination inversely affects the richness of archaeal communities in sediments (Roling et al., 2004). GeoChip hybridization also showed that soil archaeal communities decreased with the petroleum contamination (Liang et al., 2009).

2.3 Phylogenetic analysis of 16S rRNA gene clone library

Representative sequences of OTUs were submitted to Genbank database for phylogenetic analysis, and highly diverse archaeal communities were observed in three soil samples (Fig. 3). The Euryarchaeota was the dominant archaeal population with Halobacteria encompassing 97.8% of the Euryarchaeota clones, and 95.9% of the total clones.

A total of 9 clusters changed in abundance along the gradient of petroleum contamination (Fig. 4). The five dominant clusters in Soil CK are clusters A, F, G, H and I, which are related to uncultured Archaea (Figs. 3 and 4). Cluster A was 98.6%–99.3% identical with uncultured euryarchaeote clone ST-3K67A recovered from seawater (Eder et al., 2002). Cluster F was related to uncultured Halobacteriales clone ZAR26 obtained from microbial mats underlying spring water (Elshahed et al., 2004). Cluster G consists of 23 OTUs related to uncultured Haloarchaea. Cluster H consists of 4 OTUs related to uncultured Archaea. Cluster I consists of 10 OTUs related to uncultured Crenarchaea.

Clones in Soil L showed a transitional pattern between Soil CK and Soil H (Figs. 3 and 4). Clusters G and A were abundant in Soil L and Soil CK, whereas cluster D was abundant in Soil L and Soil H.

Clusters of B, C, D and E are dominant in heavily contaminated Soil H (Figs. 3 and 4). Cluster B was 98.6%–99.2% identical with *Haloferax* sp. B-1 isolated from sea salt. Cluster C was 95.0%–95.1% identical with Halobacteriaceae archaeon TBN49, which was derived from marine solar salterns. Cluster D was 96.6%–97.2% identical with *Natrinema* sp. CX2021 (Zhou et al., 2008). Cluster E was 95.1%–96.5% identical with *Natronomonas pharaonis* DSM2160, which is a haloalkaliphilic archaeon

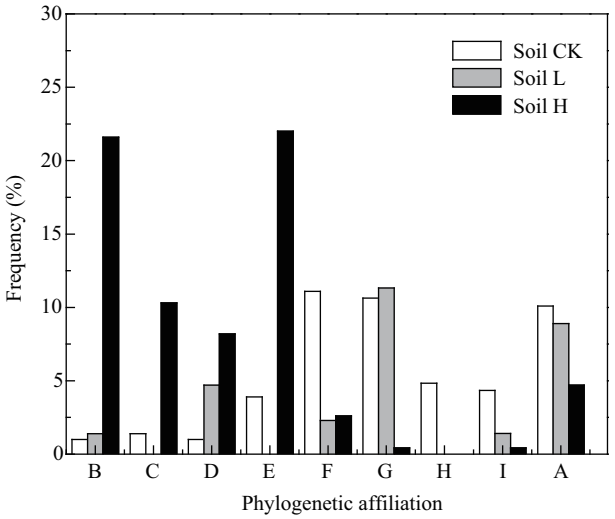

Fig. 4 Frequency of archaeal 16S rRNA gene sequences obtained from petroleum-contaminated saline-alkali soils.

(Kamekura et al., 1997). Halophilic *Haloferax* was reported to degrade alkanes and phenanthrene in saline environments (Al-Mailem et al., 2010b; Tapilatu et al., 2010), and *Haloferax* sp. D1227 could degrade aromatic substrates, including benzoate, cinnamate, and phenylpropanoate (Fu and Oriel, 1998; 1999). *Natronomonas,* a haloalkaliphilic archaeon that has a large group of genes involved in the fatty acid degradation pathway (Falb et al., 2005; Konstantinidis et al., 2007; Oren, 2006), also dramatically increased in abundance with the increase of TPH concentrations. It is believed that fatty acid degradation pathway has an important role in alkane degradation. Therefore, *Haloferax* and *Natronomonas* are likely to involve in the degradation of petroleum hydrocarbon in saline-alkali soils.

Archaeal communities were revealed by cultural-independent method in this study. Thus further research is needed to determine the degradation capacity of dominant archaeal populations with pure cultures. Despite this limitation, the findings presented in this article hint the contribution of Archaea in the natural attenuation of petroleum contamination in saline-alkali soils.

3 Conclusions

The richness of archaeal community decreases with increasing TPH concentrations in saline-alkali soils. Heavily polluted saline-alkali soil contains more clones related to hydrocarbon-degrading Archaea. Predominance of the halophilic genus of *Haloferax* and the haloalkaliphilic genus of *Natronomonas* in soils with high TPH concentrations suggests that archaeal communities could have a role in the natural attenuation of petroleum-contaminated saline-alkali soil.

Acknowledgments

This work was supported by the Knowledge Innovation Program of the Chinese Academy of Sciences (No. KZCX1-YW-06-03).

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